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Optimizing Fermentation Conditions of Recombinant *Escherichia coli* Expressing Cyclopentanone Monooxygenase

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Abstract:

Microbial Baeyer−Villiger oxidation of different substrates with cyclopentanone monooxygenase (CPMO) from *Comamonas* NCIMB 9872 was up-scaled to benchtop fermenter scale. Conditions for cell growth and biocatalyst production were optimized, and a convenient, environmentally friendly and applicable methodology for the preparation of chiral building blocks for natural product and bioactive compound synthesis was developed by applying a resin based on the concept of in situ "substrate feeding−product removal" (SFPR). Three different ketones (4-methylcyclohexanone, *rac*-3-methylcyclohexanone, and 8-oxabicyclo[3.2.1]oct-6-en-3-one) were converted in 5−15 g/L scale in a conventional bioreactor, with a volumetric productivity of up to 1 g L^{-1} h^{-1} in good to excellent yield and enantiomeric purity.

Introduction

As biocatalysis moves from laboratory to production scales, economic considerations become increasingly important.[1] High stereoselectivity and good substrate acceptance can be achieved by choosing the most appropriate enzyme. Suitable biocatalysts can be obtained by screening recombinant whole cells or isolated enzymes from the natural diversity.[2] In addition, modern molecular biological methodologies such as directed evolution or gene shuffling offer strategies to influence and optimize the performance of a biocatalytic entity.[3]

Enzyme-mediated Baeyer−Villiger oxidation offers a "green chemistry" approach for the production of chiral lactones.[4] This reaction (Figure 1), named after its discoverers Adolf von Baeyer and Victor Villiger, represents a powerful methodology in synthesis for breaking carbon−carbon bonds in oxygen-insertion process.[5] In contrast to classical protocols

Figure 1. **Baeyer−Villiger oxidation of cyclic ketones to lactones.**

of this reaction, which require potentially explosive oxidants (such as hydrogen peroxide or various peracids), display limited functional group tolerance, and lack high stereoselectivity with de novo designed chiral catalysts, biocatalysis offers a very mild, environmental friendly and stereoselective entry to this process by utilizing molecular oxygen as oxidant.[6] An increasing number of flavin-dependent Baeyer−Villiger monooxygenases (BVMOs) with a remarkably broad profile of non-natural substrates has been identified during recent years.[7,8] This led to the identification of an increasing variety of structurally diverse substrate ketones which are converted efficiently and in high stereoselectivity and which can serve as precursors for the subsequent asymmetric synthesis of natural and bioactive compounds (Figure 2).[9] Consequently, the development of an applicable and efficient up-scaling process to overcome limitations in accessibility of such chiral intermediates is becoming increasingly important to the field.[10]

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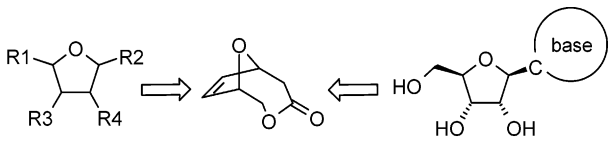

Figure 2. **Application of microbial Baeyer−Villiger oxidation in the synthesis of bioactive compounds.**

The limited stability of the most abundantly utilized Baeyer−Villiger monooxygenases together with the requirement of expensive cofactors (NADPH/$NADP^+$) has limited widespread application of enzyme-mediated Baeyer−Villiger oxidations thus far. To circumvent the obstacles of enzyme isolation and cofactor recycling, recombinant whole-cell overexpression systems were introduced in recent years. This strategy was successfully used in synthetic applications of BVMOs from various native origins, and both *Saccharomyces cerevisiae*[11] and *Escherichia coli*[12] were established as suitable host organisms.

Critical parameters in the optimization of fermentation conditions include temperature, pH, media composition, oxygen availability, efficiency of cofactor recycling, and utilization of highly productive recombinant *E.coli* strains, which have to be fine-tuned in an iterative process. Recently, some of us (R.F. and V.A.) developed a convenient and efficient solid-phase based "substrate feeding−product removal" (SFPR) concept,[13] a special case of extractive catalysis[14] and applied it to BVMO biooxidations. This approach offers a solution to several fermentation difficulties and gave impressive results since a kilogram-scale biotransformation was operated successfully.[14d] Thus, a resin serves as reservoir for both substrate and product, hence limiting both concentrations within the fermentation broth below toxic/inhibition levels. While in preceding work this concept was exclusively applied to a single Baeyer−Villiger biooxidation, we wanted to evaluate the possibility of this "two-in-one" resin-based SFPR concept as a general tool in whole-cell mediated Baeyer−Villiger fermentation processes in the present study. This included the extension to highly different substrates and the utilization of a different recombinant *E.coli*-based expression system. We chose recombinant cells producing cyclopentanone monooxygenase (CPMO) from *Comamonas* NCIMB 9872,[8b] as this enzyme displayed a remarkable substrate profile and stereoselectivity in previous studies.[15]

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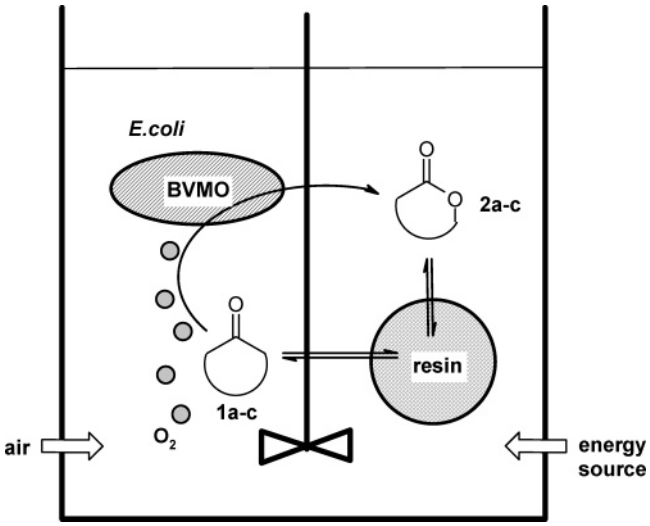

Figure 3. **Principle of in situ substrate feeding/product removal concept (SFPR).**

The ultimate goal of our ongoing research program is to reach substrate concentrations of grams per liter fermentation volume together with an easy work-up and purification procedure of the desired product for subsequent industrial scale applications.

The SFPR-Concept

The concentrations of substrate and product in the liquid phase have to be controlled during the whole fermentation process to ensure maximum cell activity. This was realized by using an adsorbent material, which was "pre"-loaded with ketone (**1a**−**c**). In the present study we exclusively used cells under growing conditions; therefore, a rapid biotransformation was mandatory as the expression system loses efficiency upon reaching the stationary phase.

An illustration of the SFPR process during the biotransformation is shown in Figure 3. First, the loaded adsorbent was added to the fermentation broth, and after a short period of time, equilibrium between the adsorbent and the aqueous phase was reached. Thus, a defined amount of substrate ketone (**1a**−**c**) can be transferred into the cell and will be oxidized by the BVMO. The product will leave the cell and can be re-adsorbed by the solid support. The equilibrium between the adsorbent and the aqueous phase has to be adjusted to concentrations below the inhibition level of the enzyme and of toxic effects to the cells. One of the most important properties of the adsorbent will be a high capacity or high load at the equilibrium concentration chosen for the biotransformation. The load is defined and measured as described in the literature.[14a]

We observed previously[14a] that resins can be classified as two types, according to the shape of the adsorption isotherm, the Γ-shape group and the S-shape group that is characterized by a better load at low concentration. Lewatit VPOC 1163, an S-shape resin with the best performance, was used for the following investigations (Figure 4).

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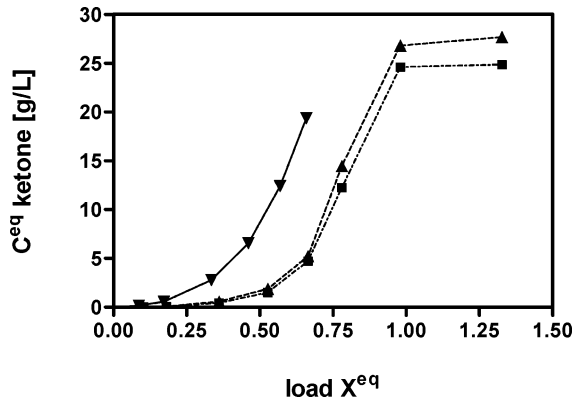

Figure 4. **Tests with S-shape resin Lewatit VPOC 1163 and three different substrates. (▲) 4-Methylcyclohexanone 1a; (■) *rac* 3-methylcyclohexanone 1b; (▼) 8-oxabicyclo[3.2.1]oct-6-en-3-one 1c.**

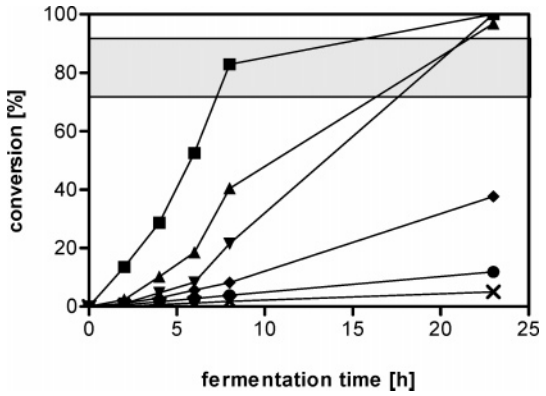

Figure 5. **Kinetic studies for 4-methylcyclohexanone 1a. (■) 3 mM; (▲) 10 mM; (▼) 20 mM; (◆) 30 mM; (●) 40 mM; (×) 50 mM.**

Results and Discussion

The main goal of this research was the optimization of the large-scale microbial Baeyer−Villiger oxidation. Due to previous shake flask experiments we decided to start the upscale of the microbial Baeyer−Villiger oxidation in a batch fermentation process under "growing" conditions. Several parameters such as oxygen saturation, time-point of induction, concentration of an auxiliary, and influence of glucose were tested. The model reaction for this process was the conversion of 4-methylcyclohexanone **1a** to the corresponding lactone.

Initially, the inhibition level for our model reaction and CPMO had to be determined. All kinetic experiments were performed in 10-mL-scale shake flask transformations under growing conditions. The obtained data showed a significant decrease of the conversion of substrate **1a** within the range of a concentration of 25−30 mM (Figure 5). Enzyme inhibition and possible toxicity of the substrate was observed. On the basis of these kinetic studies for 4-methylcyclohexanone **1a**, all fermentations were performed with a maximum concentration of 30 mM (3.36 g/L).

A first set of experiments was aimed at the optimization of the fermentation process without using a solid support reservoir. The fermentation media (1 L terrific broth TB supplemented by 200 mg/L ampicillin) was inoculated with an overnight culture (2 vol %) of the recombinant microorganism (DH5α/CPMO) at 37 °C. After 2 h of growing ($OD_{590} \approx 1$, 0.43 g/L dcw), the fermentation temperature was decreased to 25 °C, and IPTG (0.25 mM) was added to induce protein production. Subsequent addition of substrate **1a** (3.36 g, 30 mM) initiated the biotransformation under growing conditions. The results during the optimization process were compiled in Table 1.

Table 1. **Data for biooxidation of 4-methylcyclohexanone 1a under growing conditions (without solid support reservoir)**

experiments	1	2	3	4	5
substrate c [mM]	30	30	30	30	30
T [°C]	25	25	25	25	25
pH	7	7	7	7	7
O_2 sat. [%]	100	30	100	50	100
glucose [g/L]	−	−	4	4	4
fermentation time [h]	21	22	23	21	23
β-cyclodextrin [mol %]	−	−	−	−	10
calculated yield [%]	56	63	72	84	81

The oxygen saturation was set at 100%, and the progress of the fermentation was determined by GC analysis with an internal standard to calculate the absolute yield of the fermentation process at any time without product isolation. The fermentation reached complete conversion after 21 h, however, with a moderate 55% calculated overall yield. Due to the loss of 45% of starting material and the volatility of the substrate, we decided to decrease the oxygen saturation to avoid evaporation of the starting material. The second experiment was performed under the same reaction conditions with an oxygen saturation of 30%. A slight increase in yield to 63% was observed. A substantial increase in efficiency of the biooxidation was achieved by addition of glucose (4 g/L) after induction of protein production, with an increase in yield to 72%. An acceptable tradeoff between sufficient aeration (to ensure both biooxidation as well as microbial growth) and minimized ketone loss (due to the air flow) was found when running the fermentation with oxygen saturation at 50% and addition of glucose (84% yield). Finally, the influence of a β-cyclodextrin additive was tested, which may act either as transportation auxiliary for the substrate into the cell or as an additional reservoir for the substrate within the fermentation broth by a host−guest interaction. With an oxygen saturation of 100%, 4 g/L of glucose, and 10 mol % of β-cyclodextrin a total yield of 81% was obtained. Nevertheless, the process is limited to a final substrate concentration of 3.36 g/L (30 mM).

Most of the critical aspects of the above set of experiments could be overcome upon applying the SFPR concept. The effect of the non-natural substrate on the biocatalyst (recombinant whole cells) was reduced to a minimum, and therefore the problems of toxicity, enzyme stability, and volatility were solved.

Three different substrates were transformed by applying the SFPR procedure described above. Commercially available prochiral (4-methylcyclohexanone, **1a**) and racemic (*rac*-3-methylcyclohexanone, **1b**) ketones were used to prove the concept and compare SFPR fermentations to previously reported results for CPMO biooxidations. CPMO is able to give antipodal lactone **2a** compared to the majority of other

BVMOs.[15,16] Ketone **1b** is transformed in a regioselective process to the proximal lactone (**2b-prox**). The attractive prochiral precursor 8-oxabicyclo[3.2.1]oct-6-en-3-one (**1c**) represented our benchmark experiment using a highly polar and structurally demanding substrate.

Based on the above results the critical limitation of the biotransformation seems to be toxicity to the whole cells eventually accompanied by enzyme inhibition. As the substrate concentration (**1a**) approached 30 mM (3.36 g/L), a significant decrease of cell growth was observed. Such stagnation in biomass consequently results in a slow biooxidation during the fermentation process due to low amount of active enzyme. As we wanted to maximize the amount of biomass, in the subsequent set of experiments implementing the SFPR concept a different biotransformation strategy was chosen. Under growing conditions the biotransformation took place at the early log phase of the growth of the cells. Biotransformations with "nongrowing" cells were investigated in the very late log phase or the beginning of the stationary phase. Therefore, the growth of the cells was continued at 37 °C under 5 vvm of air until an optical density of 7−8 was reached (or when the change in OD_{590} increased by less than 0.5 au over a 30-min period). Using this strategy, cell growth was not decreased by the presence of a toxic substrate. As soon as the maximum amount of biomass was produced, the fermentation temperature was decreased to 25 °C and protein production was induced by addition of IPTG (0.25 mM). At this temperature the cell division rate was slowed significantly. After 1 h of induction 4 g/L of glucose was added. Another hour later cells were sampled and tested for their biooxidation activity. These tests were performed with a model reaction using the highly reactive ketone bicyclo[3.2.0]hept-2-en-6-one known from other BVMO biotransformations in a shake flask experiment.[14a] A standard solution of bicyclo[3.2.0]hept-2-en-6-one in ethanol was added to 20 mL of the growing culture up to a concentration of 0.5 g/L. Complete conversion after 1 h has to be achieved for cells with a high biooxidation activity.

Subsequently, the preloaded resin was added, and the biotransformation was monitored by GC/MS. The resin and the aqueous fermentation solution were extracted separately with dichloromethane or ethyl acetate supplemented by an internal standard (methylbenzoate) to monitor the progress of the reaction.

Based on our results from resin and kinetic studies, 10 g/L of 4-methylcyclohexanone **1a** were successfully converted in a first attempt by CPMO-producing growing cells to the corresponding lactone **2a** (Figure 6). The biotransformation was performed under the same reaction conditions as outlined in experiment 5 (Table 1) and gave lactone **2a** after 26 h with 77% yield (Figure 7). In a second optimization experiment we performed the same biotransformation with 15 g/L 4-methylclohexnone **1a** under non-growing conditions and obtained 86% of the desired lactone **2a** in only 20 h of fermentation time.

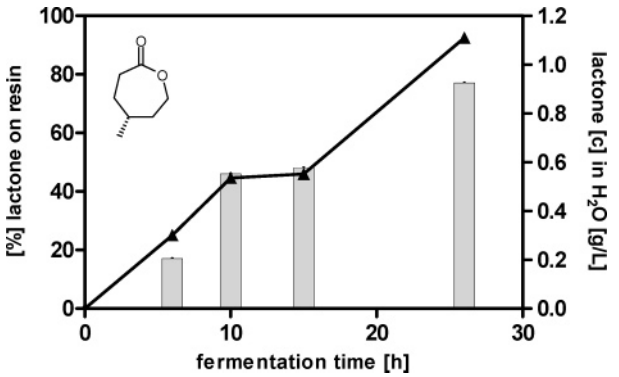

Figure 6. **Fermentation of 10 g/L 4-methylcyclohexanone 1a with CPMO growing cells; (▲) g/L lactone in aqueous phase; (Shaded area in columns) percent lactone of organic compounds adsorbed to the resin.**

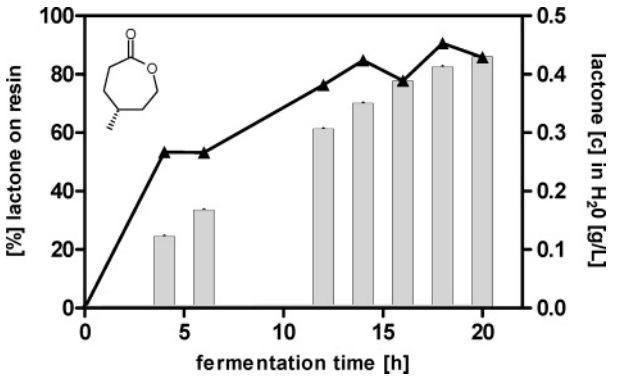

Figure 7. **Fermentation of 15 g/L 4-methylcyclohexanone 1a with CPMO non-growing cells; (▲) g/L lactone in aqueous phase; (Shaded area in columns) percent lactone of organic compounds adsorbed to the resin.**

This represents a 5-fold increase in substrate concentration and an improved yield compared to results from growing cell fermentation conditions without using the SFPR concept. The combination of non-growing cells and the SFPR concept turned out to be the best conditions for a biooxidative whole-cell Baeyer−Villiger process. No problems with respect to substrate volatility were observed. The performance of the CPMO-producing cells was in the range previously reported in the literature on an overexpression system for cyclohexanone monooxygenase (CHMO).[17] The fastest biotransformation and therefore the highest BVMO activity were determined within the first 12 h (60% conversion), and afterwards a moderate loss of activity was observed.

The second example for the "SFPR" concept was demonstrated with racemic 3-methylcyclohexanone **1b** which is oxidized regioselectively to the proximal lactone **2b-prox** (Figure 8).[18]

Biotransformation with 15 g/L *rac*-3-methylcyclohexanone was successfully performed using the SFPR concept (Figure 9). Again, the majority of starting material (80%) was consumed within the first 10 h of the biotransformation. The corresponding lactone **2b-prox** was isolated in 96% yield after 16 h of fermentation time.

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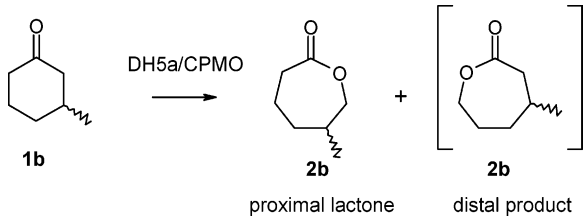

Figure 8. **Regioselective Baeyer−Villiger oxidation of *rac*-3-methylcyclohexanone 1b by CPMO yielding only proximal lactone 2b-prox.**

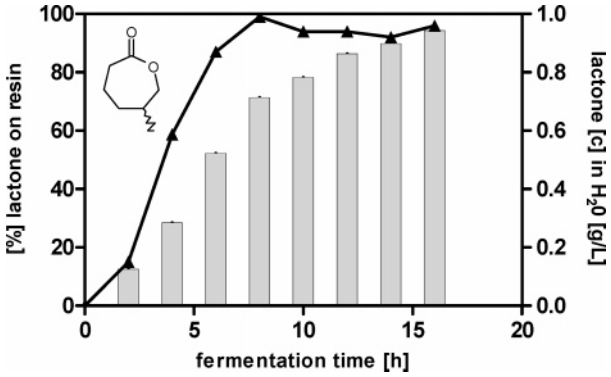

Figure 9. **Fermentation of 15 g/L *rac*-3-methylcyclohexanone 1b with CPMO non-growing cells; (▲) g/L lactone in aqueous phase; (Shaded area in columns) percent lactone of organic compounds adsorbed to the resin.**

Ketone **1c** (8-oxabicyclo[3.2.1]oct-6-en-3-one) represents an interesting starting material for biooxidative desymmetrization, offering access to lactone **2c** as versatile platform for subsequent synthetic transformations (Figure 2). We are currently evaluating the potential of this intermediate in the synthesis of a variety of natural products and bioactive compounds. Ketone **1c** was synthesized via a facile [3 + 4] cycloaddition reaction and subsequent debromination.[19] The prochiral compound possesses a relatively high water solubility and is only moderately stable at slightly elevated temperatures (>35°C) and under acidic conditions. Previous attempts failed to increase the fermentation concentration of ketone **1c** to more than 2.0 g/L (70% isolated yield) because of substrate inhibition. The substrate showed a very slow kinetics, and therefore highly active cells and long reaction times are required. Due to investigations with the SFPR concept we present an effective methodology for the biooxidation of this kind of substrate. The results are summarized in Figure 10. Interestingly, after the first 14 h only 29% conversion of the starting material was observed. The following 22 h of the biotransformation yielded in total 78% of lactone **2c**. In contrast to previous experience in shake flask experiments, no hydrolytic degradation of the lactone product was observed upon prolonged fermentation times. The addition of β-cyclodextrin (10 mol %) was mandatory for a successful biotransformation.

We demonstrated that the microbial Baeyer−Villiger oxidation can be performed in multiple-gram scale using the SFPR concept. Together with previous studies on CHMO, this technique can be regarded as a general tool to improve

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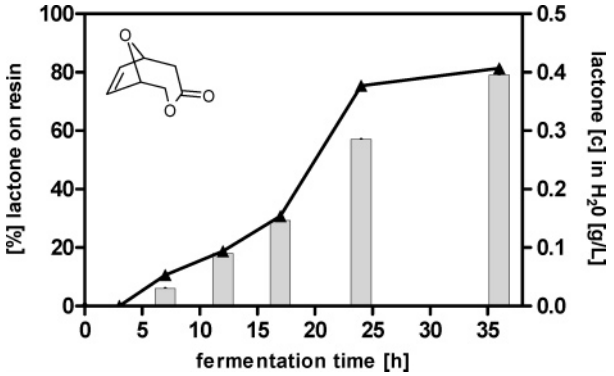

Figure 10. **Fermentation of 5 g/L 8-oxabicyclo[3.2.1]oct-6-en-3-one 1c with CPMO non-growing cells; (▲) g/L lactone in aqueous phase; (Shaded area in columns) percent lactone of organic compounds adsorbed to the resin.**

the performance of recombinant whole-cell expression systems for BVMOs. In particular, the method circumvents toxicity and inhibition problems and minimizes losses on volatile substrates. Currently, a further increase of substrate concentration by combination of the SFPR concept with "stationary phase" cells in minimal media and the application of repeated fermentation cycles is under investigation. With progress in the development of a tool-box of BVMOs, we are confident of establishing the microbial Baeyer−Villiger oxidation as a standard technique in synthetic chemistry for the production of chiral lactones as versatile intermediates in bioactive compound preparation.

Experimental Section

Unless otherwise noted, chemicals and microbial growth media were purchased from commercial suppliers and used without further purification. All solvents were distilled prior to use. General conversion control and examination of purified products were performed with ThermoFinnigan GC 8000 TOP/MS Voyager using a standard capillary column BGB-5 (30 m × 0.32 mm i.d.). Enantiomeric excess was determined via GC using a BGB-175 column (30 m × 0.25 mm i.d., 0.25 μm film) on a ThermoQuest Trace GC 2000, and data matched with previous reports. Biotransformations (aqueous phase and resin) were sampled for GC/MS analysis by mixing 500 μL of aqueous reaction mixture or a sample of the resin with 500 μL of dichloromethane containing 2−3 mM methyl benzoate (internal standard). After vortex mixing and centrifugation the organic layer was analyzed by GC/MS. Glucose concentrations were determined with *Roche Accu Chek-go*. LB medium used for cell growth contained bacto-peptone (10 g/L), bacto-yeast extract (5 g/L), sodium chloride (10 g/L), and 50 mg/mL of ampicillin. Agar (15 g/L) was added to solidify media for plates. TB medium contained bacto-tryptone (12 g/L), bacto-yeast extract (24 g/L), glycerol (4 mL/L), $K_2HPO_4 \cdot 3H_2O$ (16.4 g/L); KH_2PO_4 (2.3 g/L).

Fresh plates were streaked weekly from glycerol stocks on solid LB medium supplemented with ampicillin. A value of 0.43 g/L dcw per OD_{590} unit was used to calculate biomass concentrations from optical density measurements. A New Brunswick Bioflow 110 fermenter equipped with pH probe,

oxygen probe, flow controller, and temperature control was used. Monitoring of all fermentation parameters was performed using the Biocommand Plus 3.30 software by New Brunswick.

Standard Procedure for 4-Methylcyclohexanone 1a Oxidation by "Growing" Cells. A New Brunswick Bioflow 110 fermenter containing 1 L of sterile TB medium supplemented with 200 mg/L ampicillin was inoculated with 20 mL (2 vol %) of overnight culture of DH5α/CPMO grown on LB medium (50 mg/mL ampicillin). The 1-L culture was grown at 37 °C with air flow of 1 L min^{-1} and stirring rates at 500 rpm. A pH of 7.00 ± 0.05 was kept constant by adding 3 N NaOH or 3 N H_3PO_4 automatically. When the culture density reached approximately 0.43 g/L dcw, the temperature was decreased to 25 °C, and IPTG was added to a final concentration of 0.25 mM. Finally, pure 4-methylcyclohexanone **1a** (3.36 g, 30 mM) was added to the fermentation culture. The oxygen saturation was maintained by adjusting the stirring rate (250−400 rpm). Amounts of 4-methylcyclohexanone **1a** and corresponding lactone **2a** were determined periodically by GC/MS.

Standard Procedure for Baeyer−Villiger Oxidation by "Non-growing" Cells. A New Brunswick Bioflow 110 fermenter containing 1 L of sterile TB medium supplemented with 200 mg/L ampicillin was inoculated with 20 mL (2 vol %) of overnight culture of DH5α/CPMO grown on LB medium (50 mg/mL ampicillin). The temperature was maintained at 37 °C, and the pH was kept constant at 7.00 ± 0.05 by adding 3 N NaOH or 3 N H_3PO_4 automatically. The 1 L culture was grown with an air flow of 5 L min^{-1} and stirring rates at 500 rpm. The growth was continued until the culture density reached 3.01−3.44 g/L dcw and the temperature was decreased to 25 °C. IPTG was added to a final concentration of 0.25 mM, and after an additional hour the fermentation culture was supplemented with 4 g/L glucose (20% sterile solution). Two hours after induction 20 mL of cell culture was taken, and activity tests were performed. After passing the activity tests the preloaded resin and any additives were added. The glucose level was measured periodically as the bioconversion progressed.

Baeyer−Villiger Oxidation of 4-Methylcyclohexanone 1a: Non-growing Conditions. Cells were grown in a 1-L benchtop fermenter as described above. Preloaded resin (4-methylcyclohexanone, 15 g, 133 mmol/75 g wet Lewatit VPOC 1163 in 100 mL LB_{Amp} medium) adjusted to load $X^{eq} = 0.4$, was added to the fermentation culture.

All process conditions were maintained at the same values as those described above. GC samples were taken periodically. After nearly complete conversion (86%) or significant decrease in the activity of CPMO (20 h) the resin was filtered off and extracted with dichloromethane continuously overnight. The aqueous solution was extracted in the same manner (overall yield: 80%, 11.7 g, 44% ee[9a]).

Biooxidation of *rac*-3-Methylcyclohexanone 1b. All process conditions—growing temperature, pH value, agitation, aeration, and glucose level—were maintained as described above. *rac*-3-Methylcyclohexanone (15 g, 133 mmol) was preloaded on Lewatit VPOC 1163 (75 g wet resin; load $X^{eq} = 0.4$). After 16 h of fermentation time nearly complete conversion (90%, 14.7 g, *rac*[18]) was determined. The product isolation was performed as described above.

Bioconversion of 8-Oxabicyclo[3.2.1]oct-6-en-3-one 1c. According to the general procedure for non-growing cells 8-oxabicyclo[3.2.1]oct-6-en-3-one was biooxidized to the corresponding lactone **2c**. Ketone **1c** (5 g, 40 mmol) was dissolved in ethanol (10 mL) and was subsequently added to the resin (50 g wet resin, load $X^{eq} = 0.2$) and 100 mL of LB_{Amp}. β-Cyclodextrin (10 mol %) and the substrate−resin mixture were added to the fermentation broth. After 36 h, the desired lactone **2c** was isolated in 70% (3.1 g, 95% ee[15]) yield.

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